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MAIL STOP APPEAL BRIEF-PATENTS
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Re: U. S. Patent Application Serial No. 10/806,494 entitled *Methods for Preventing Photodamaged Skin by Administering Selegiline or Desmethylselegiline* by Mark G. Resnick
(Our Ref: SOM700/4-9(A)8CON2US/64001)

Dear Sir:

Enclosed for filing in the above-referenced patent application are the following:

1. Appeal Brief in triplicate, including Appendix A (14 pages each), Exhibit A, and Exhibit B;

2. Credit Card Payment Form ($500.00); and

4. Postcard.

If the referenced authorization is inadvertently omitted or deficient, or should an overpayment be included herein, the Commissioner is authorized to appropriately deduct or credit the requisite amount from Vinson & Elkins L.L.P. Deposit Account No. 22-0365/SOM700/4-9(A)8CON2US/64001).

Very truly yours,

Margaret Sampson

Margaret J. Sampson

MJS/cp
Enclosures
cc: Dr. Al Azzaro (w/encls.)
Nanette W. Mantell, Esq. (w/encls.)
Marya Breig, Docket Coordinator (w/o encls.)
642682_1.DOC

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:
Mark G. Resnick

Serial No.: 10/806,494

Filed: March 3, 2004

For: METHODS FOR PREVENTING PHOTODAMAGED SKIN BY ADMINISTERING SELEGILINE OR DESMETHYLSELEGILINE

Group Art Unit: 1615

Examiner: L.S. Channavajjala

Atty. Dkt. No.: SOM700/4-009(A)8CON2/64001

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APPEAL BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In support of the appeal from the final rejection dated May, 27, 2005, Appellant now submits this Brief.

I. REAL PARTY IN INTEREST

The real party in interest of the patent application that is the subject of this appeal is the assignee, Somerset Pharmaceuticals, Inc.

II. RELATED APPEALS AND INTERFERENCES

No other appeals or interferences are known to Appellant that will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 31-53 are pending in the instant application and are the subject of this appeal. Claims 1-30 have been previously cancelled without prejudice. All claims stand rejected by the Examiner. A final rejection was issued on May, 27, 2005 rejecting all pending claims. A Notice of Appeal was filed on August 29, 2005 to appeal the final rejection of Claims 31-53. A copy of Claims 31-53 on appeal is attached hereto as Appendix A.

IV. STATUS OF AMENDMENTS

No amendments have been filed after final rejection, and no amendments are submitted with this appeal brief.

V. SUMMARY OF INVENTION

The invention defined by the rejected claims relates to a method of reducing photodamage to skin cells through the administration of selegiline and/or desmethylselegiline in an amount effective to reduce apoptosis of skin cells exposed to electromagnetic or ionizing radiation (*see, e.g.*, p.8, lines 19-20). In addition, the invention relates to a method of treating a subject for photodamaged skin through the

administration of selegiline and/or desmethylselegiline to the skin in an amount effective to reduce oxidative damage resulting from exposure to electromagnetic or ionizing radiation (*see, e.g.*, p.4, lines 8-13 and 20). Although the methods of the present disclosure are directed to the administration of effective amounts of selegiline or desmethylselegiline by any route, the methods of the invention preferably include administration of selegiline and desmethylselegiline as a topical composition, for example in the form of a cream, gel, transdermal patch, salve, lotion, or spray (*see, e.g.*, p.5, lines 4-7).

Selegiline and desmethylselegiline may be preferably administered as a topical composition comprising selegiline and/or desmethylselegiline at a concentration of between 1 x 10^{-11} moles/liter and 1 x 10^{-3} moles/liter (*see, e.g.*, p.5, line 15). The present disclosure includes the administration of both enantiomeric forms of desmethylselegiline, *i.e.*, the R(-) enantiomeric form of desmethylselegiline or the S(+) enantiomeric form of desmethylselegiline (*see, e.g.*, p.5, lines 9-11). Preferably, a racemic mixture of the two may also be used in the claimed invention (*see, e.g.*, p.5, lines 7-11). Selegiline and desmethylselegiline may be administered as either the free base or as a pharmaceutically acceptable salt form (*see, e.g.*, p.8, lines 2-4).

The R(-) enantiomer of desmethylselegiline may be used in the substantial absence of the S(+) enantiomer of desmethylselegiline, or vice versa (*see, e.g.*, p.5, lines 10-11). An enantiomer is substantially absent if it constitutes less than 10% of the combined desmethylselegiline enantiomers (*see, e.g.*, p.5, lines 11-12). Compositions administered in the methods of the presently claimed invention may contain water,

suspending agents, thickeners, humectants, preservatives, emollients, emulsifiers and film formers (*see, e.g.*, p.5, lines 12-13).

VI. ISSUES

The issues for determination in this appeal are:

1) Whether claims 31-53 are unpatentable under 35 U.S.C. §103(a) as obvious over Tatton et al. (Neurology, 1996) in view of U.S. Patent No. 5,744,499 to Quash et al.
2) Whether claims 31-53 are unpatentable under 35 U.S.C. §103(a) as obvious over U.S. Patent No. 5,744,499 to Quash et al. in view of Tatton et al. (Neurology, 1996).
3) Whether claims 31-53 are unpatentable under 35 U.S.C. §103(a) as obvious over U.S. Patent No. 5,783,606 to Tatton in view of U.S. Patent No. 5,744,499 to Quash et al. and in view of Tatton et al. (Neurology, 1996).

VII. GROUPING OF CLAIMS

Claims 31-53 are grouped together.

VIII. ARGUMENT

A. Rejections Based on 35 U.S.C. § 103 are Overcome

1. The Rejections

In the Final Office Action ("Final Action") issued May 27, 2005, claims 31-53 were rejected under 35 U.S.C. § 103(a) over Tatton et al. (Neurology, 1996) in view of U.S. Patent No. 5,744,499 to Quash et al. (Quash); over Quash in view of Tatton et al.; or over U.S. Patent No. 5,783,606 to Tatton (Tatton '606) in view of Quash and Tatton et al. The Examiner states that "Tatton et al. teach deprenyl (same as selegiline) for reducing neuronal apoptosis caused by oxidative free radical damage and the reduction is mediated by a principal metabolite of deprenyl, desmethyldeprenyl (same as desmethylselegiline)." The Examiner also states that Tatton `606 "teaches deprenyl and desmethyldeprenyl compounds for the treatment of glaucoma" and "administering deprenyl compositions in the form of sprays, liquids, gels, pastes etc., for oral, nasal, topical, or other routes." Finally, the Action states that Quash "teaches modulation of apoptosis (induce or suppress) as a mechanism to prevent or provide treatment for photoinduced or chronological aging of skin and other related skin conditions."

2. The Examiner Has Not Established a Prima Facie Case of Obviousness

The Examiner cites the above references to assert that it would have been obvious "for one of ordinary skill in the art at the time of the instant invention to use the anti-apoptotic compounds (deprenyl and desmethyldeprenyl) of Tatton et al.(Neurology) for inhibiting or suppressing apoptosis in several dermal or epidermal conditions such as aging because Quash teaches that skin aging basically result from malfunctioning of skin

mechanisms, especially due to apoptosis and suggests any species capable of modulating apoptosis can also prevent aging and its signs such as wrinkles."

But as Appellant demonstrates below, the teaching by Quash that any species capable of modulating apoptosis can also prevent aging and its signs is simply not true. Based on the skilled person's knowledge of the art (*i.e.*, knowledge of at least one anti-apoptotic compound that does not protect skin against photodamage), this person would have understood the fault of the premise stated in Quash, and therefore would not find the presently claimed subject matter obvious.

When setting forth an obviousness rejection, the MPEP clearly indicates that it is the Examiner who "bears the initial burden of factually supporting any *prima facie* conclusion of obviousness." MPEP § 2142. The MPEP sets forth that to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be a suggestion or motivation to combine the reference teachings either in the references themselves or in the general knowledge of one of ordinary skill in the art, (2) there must be a reasonable expectation of success, and (3) the references when combined must teach or suggest all the claim limitations. MPEP §§ 2142 & 2143. The Examiner bears the initial burden of factually supporting each of the three elements in order to make out a *prima facie* case of obviousness.

While Appellant asserts that the obviousness rejection in the Final Action fails on all three criteria to establish a *prima facie* case of obviousness, for the sake of efficiency Appellant will focus on how the rejection fails under the second criteria: *based on the combined references, there is no reasonable expectation of success*. Since the Examiner

has not met her burden of showing a *prima facie* case of obviousness, Appellant is under no obligation to submit evidence of nonobviousness.

2. The Obviousness Rejection Does Not Establish a Reasonable Expectation of Success

The Examiner bears the initial burden of factually supporting a *prima facie* case of obviousness, which necessarily requires that the Examiner factually establish a reasonable expectation of success when combining the teachings of the references. MPEP § 2142. The Examiner however, cannot show the required "reasonable expectation of success," because one of skill in the art would not expect *any anti-apoptotic compound to treat photodamaged skin*, despite the suggestion made in Quash.

The Examiner relies on Quash to show a reasonable expectation of success: "Quash teaches that skin aging basically result (sic) from malfunctioning of skin mechanisms, especially due to apoptosis and suggests any species capable of modulating apoptosis can also prevent aging and its signs such as wrinkles." From this statement the Examiner concludes that "one of an ordinary skill in the art would have incorporated the compounds of Tatton et al. in the composition Quash and use for treating and/or combating photoinduced or chronological aging of the skin by modulating apoptosis because Tatton et al. suggests that the claimed compounds have the ability to reduce oxidative free radical initiated apoptosis." Tatton '606 is cited by the Examiner to teach the use of "deprenyl and desmethyldeprenyl compounds for the treatment of glaucoma" and the administration of deprenyl in various forms for oral, nasal, topical, or other routes of administration.

One of skill in the art would reject the suggestion made in Quash (and relied on by the Examiner for the obviousness rejection) because Quash does not provide sufficient factual support for the proposition that all anti-apoptotic agents would be expected to be effective in treating photodamage. For example, Quash is narrowly drawn to modulating apoptosis with methional, malondialdehyde, or factors influencing the intracellular concentrations of methional or malondialdehyde. One of skill in the art would not consider the disclosure of only two anti-apoptotic agents to support the generalization made in Quash. Quash simply does not disclose a representative number of species to support this broad suggestion. Thus, the Examiner's reliance on Quash to factually establish a reasonable expectation of success by discussing the use of two compounds for modulating apoptosis out of the broad genus of anti-apoptotics is misplaced. "The fact that a claimed species or subgenus is encompassed by a prior art genus is not sufficient by itself to establish a *prima facie* case of obviousness." MPEP § 2144.08 (II).

In addition to the minimal experimentation by Quash to support the broad suggestion relied upon by the Examiner, one of skill in the art would be aware that at least one well known anti-apoptotic compound, beta-carotene, has not been convincingly demonstrated in most clinical studies to protect against skin photodamage: "Undoubtedly, beta carotene is an important nutrient with powerful biological effects. Nevertheless, clinical studies as a whole have failed to persuade of an important role of beta-carotene as a photoprotector." (See Biesalski et al., Arch. Biochem. Biophys. 389(1):1-6 (2001), attached hereto as Exhibit A ("Clinical trials demonstrated protective effects of beta-carotene against acute skin reactions [ ], but they failed to show any prevention of chronic photodamage [ ]. At present, both clinical and experimental data

are highly inconsistent and some recent results also indicate the existence of potentially harmful effects of beta-carotene in UV-irradiated skin [ ]."). This knowledge would stop one of skill in the art from concluding that *any* anti-apoptotic compound can "prevent aging and its signs such as wrinkles." MPEP § 2142 requires that the Examiner show a *reasonable* expectation of success, not a mere possibility of success.

Beta-carotene has been shown in hepatic and brain cell lines to be an anti-apoptotic compound (Bagchi et al., Gen. Pharmac. 30(5):771-76 (1998), attached hereto as Exhibit B; see also Ortmann *et al.*, Radiat. Res. 161(1):48-55 (2004) ("When given prior to irradiation, beta-carotene and vitamin E reduced the amount of radiation-induced apoptosis significantly...")). For example, Bagchi et al. found that beta-carotene was able to reduce TPA-induced hepatic and brain DNA fragmentation by 11% (p. 774). Since "[f]ragmentation of nuclear DNA is a biochemical hallmark of apoptosis," this finding demonstrates that beta-carotene has anti-apoptotic properties (p. 774).

Since the anti-apoptotic compound beta-carotene has not been shown to protect against skin photodamage, the suggestion by Quash relied on by the Examiner as the basis of the obviousness rejection is refuted. One of skill in the art would not have had a reasonable expectation of success in treating or promoting the healing of photodamaged skin with *any* anti-apoptotic compounds, therefore, one of skill in the art would not have a reasonable expectation that selegiline or desmethylselegiline would have this claimed activity based solely on the knowledge that selegiline and desmethylselegiline have anti-apoptotic activity.

Given the faulty premise of Quash, the Examiner has not provided factual support establishing a reasonable expectation of success in utilizing desmethylselegiline and/or

selegiline to treat photodamaged skin. Therefore, The Examiner has not met her burden to establish a *prima facie* case of obviousness.

IX. CONCLUSION

Appellant respectfully submits that based on the foregoing observations and arguments, all pending claims listed in the Appendix A are non-obvious. It is therefore respectfully requested that the Board overturn the Examiner's rejections.

Respectfully submitted,

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APPENDIX A

Claims 1-30 (Canceled).

Claim 31. (Previously presented) A method of reducing photodamage to skin cells of a subject comprising administering a composition comprising selegiline and/or desmethylselegiline to the subject in an amount effective to reduce apoptosis of skin cells exposed to electromagnetic or ionizing radiation.

Claim 32. (Previously presented) The method of claim 31, wherein the composition is a topical composition.

Claim 33. (Previously presented) The method of claim 32, wherein the topical composition is in a form selected from the group consisting of a cream, gel, transdermal patch, salve, lotion, and spray.

Claim 34. (Previously presented) The method of claim 32, wherein the topical composition comprises selegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 35. (Previously presented) The method of claim 32, wherein the topical composition comprises desmethylselegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 36. (Previously presented) The method of claim 31, wherein the composition comprises desmethylselegiline in the form of its R(-) enantiomer and the S(+) enantiomer is substantially absent.

Claim 37. (Previously presented) The method of claim 31, wherein the composition comprises desmethylselegiline in the form of its S(+)enantiomer and the R(-) enantiomer is substantially absent.

Claim 38. (Previously presented) The method of claim 31, wherein the composition further comprises a diluent or carrier comprising one or more compounds selected from the group consisting of water, suspending agents, thickeners, humectants, preservatives, emollients, emulsifiers, and film formers.

Claim 39. (Previously presented) The method of claim 31, wherein the selegiline is administered as the free base.

Claim 40. (Previously presented) The method of claim 31, wherein the selegiline is administered as a pharmaceutically acceptable acid addition salt.

Claim 41. (Previously presented) The method of claim 31, wherein the desmethylselegiline is administered as the free base.

Claim 42. (Previously presented) The method of claim 31, wherein the desmethylselegiline is administered as a pharmaceutically acceptable acid addition salt.

Claim 43. (Previously presented) A method of treating a subject for photodamaged skin, wherein the photodamage results from exposure to electromagnetic or ionizing radiation, comprising administering a topical composition comprising selegiline and/or desmethylselegiline to the skin of the subject in an amount effective to reduce oxidative damage resulting from exposure to electromagnetic or ionizing radiation.

Claim 44. (Previously presented) The method of claim 43, wherein the topical composition is in a form selected from the group consisting of a cream, gel, transdermal patch, salve, lotion, and spray.

Claim 45. (Previously presented) The method of claim 43, wherein the topical composition comprises selegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 46. (Previously presented) The method of claim 43, wherein the topical composition comprises desmethylselegiline at a concentration of between 1×10^{-11} moles/liter and 1×10^{-3} moles/liter.

Claim 47. (Previously presented) The method of claim 43, wherein the topical composition comprises desmethylselegiline in the form of its R(-) enantiomer and the S(+) enantiomer is substantially absent.

Claim 48. (Previously presented) The method of claim 43, wherein the topical composition comprises desmethylselegiline in the form of its S(+)enantiomer and the R(-) enantiomer is substantially absent.

Claim 49. (Previously presented) The method of claim 45, wherein the topical composition further comprises a diluent or carrier comprising one or more compounds selected from the group consisting of water, suspending agents, thickeners, humectants, preservatives, emollients, emulsifiers, and film formers.

Claim 50. (Previously presented) The method of claim 45, wherein the selegiline is administered as the free base.

Claim 51. (Previously presented) The method of claim 45, wherein the selegiline is administered as a pharmaceutically acceptable acid addition salt.

Claim 52. (Previously presented) The method of claim 45, wherein the desmethylselegiline is administered as the free base.

Claim 53. (Previously presented) The method of claim 45, wherein the desmethylselegiline is administered as a pharmaceutically acceptable acid addition salt.

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MINIREVIEW

UV Light, Beta-carotene and Human Skin—Beneficial and Potentially Harmful Effects

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Solar radiation is one of the most important environmental stress agents for human skin, causing sunburn, premature skin aging, and skin cancer. Beta-carotene is discussed to protect against photooxidative stress and thus prevent skin damage. Though beta-carotene has been successfully used against photosensitivity in patients with erythropoietic protoporphyria, its beneficial potential in normal skin is still uncertain. A number of experimental studies indicate protective effects of beta-carotene against acute and chronic manifestations of skin photodamage. However, most clinical studies have failed to convincingly demonstrate its beneficial effects so far. Nevertheless, intake of oral beta-carotene supplements before sun exposure has been recommended on a population-wide basis. Recent studies on skin cells in culture have revealed that beta-carotene acts not only as an antioxidant but also has unexpected prooxidant properties. At present, there is an ongoing debate on the protective or potentially harmful role of beta-carotene in human skin. © 2001 Academic Press

Key Words: **UV; carotenoids; beta-carotene; skin; oxidative stress; antioxidant; prooxidant.**

Beta-carotene has repeatedly been called a "sun protectant" and been credited with preventing solar damage to skin. Consequently, intake of oral supplements in times of increased sun exposure has been suggested to be beneficial (1–3) and is now very popular among sun seekers. The essential function of carotenoids protecting cells against photosensitized reactions was first hypothesized in the 1950s (4). Later, beta-carotene was found to prevent endogenous (chlorophyll) and exogenous photosensitization in bacteria, algae, and higher plants (5). Moreover, beta-carotene protected mice treated with hematoporphyrin (6) and humans suffering from photosensitivity to visible light (7). High-dose oral administration of beta-carotene has become a useful tool for therapy in patients with erythropoietic protoporphyria (EPP)[2] (8). This has lead to the suggestion that beta-carotene might also have protective properties in normal skin and thus prevent solar damage.

Overexposure to sunlight provokes acute sunburn reaction which clinically manifests itself as erythema. Chronic exposure to sun leads to premature skin aging ("photoaging") and increases the risk of both cutaneous melanoma and nonmelanoma skin cancer (NMSC) (9). Solar radiation has a strong oxidative component, and photooxidative stress has been directly linked to the onset of skin photodamage, as extensively reviewed by Fuchs (10). UVB radiation (280–320 nm) mainly damages DNA directly, due to an overlap with the absorption spectrum (11), and thus comprises a strong mutagenic potential. At the same time, it also has an oxidative component (12, 13). UVA radiation (320–400 nm), which contributes to up to 95% of total UV exposure (14), is not absorbed by DNA but it is a strong oxidant and considered the most important source of oxidative stress in human skin (15, 16).

The proposed beneficial effects of beta-carotene in skin have been mainly attributed to its antioxidant properties (2, 17–21). Experimental studies repeatedly found protection against UV-induced photodamage

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[2] Abbreviations used: EPP, erythropoietic protoporphyria; NMSC, nonmelanoma skin cancer; MED, minimal erythemal dose; HO1, heme oxygenase-1; DMBA, *N*-Benzyldime thylamin; TPA, Phorbol-12-myristat-13-acetate.

0003-9861/01 $35.00

Exhibit A

such as erythema, immunosuppression, or skin cancer (22-26), though some authors found no beneficial effects (23, 27–30). So far, no clinical studies have been able to confirm these promising experimental results clearly (10). Clinical trials demonstrated protective effects of beta-carotene against acute skin reactions (2, 17, 31–33), but they failed to show any prevention of chronic photodamage (34–36). At present, both clinical and experimental data are highly inconsistent and some recent results also indicate the existence of potentially harmful effects of beta-carotene in UV-irradiated skin (37, 38). This review will briefly discuss the role of beta-carotene in human skin and its possible potential in protecting against the deleterious effects of solar radiation.

BETA-CAROTENE IN HUMAN SKIN

Carotenoids are part of the coloring system in human skin (39). Basal levels of beta-carotene in skin are usually rather low and were detected at ~0.03 to 0.4 nmol/g in wet tissue (1, 40) or at ~1.5 nmol/g when subcutaneous fat was included in samples (41). After consumption of a diet rich in carotenoids or oral supplementation, skin levels of beta-carotene are likely to increase, up to 17-fold over basal levels (1), which clinically manifests as yellowish complexion ("carotenodermia") (17, 31, 42–44).

In skin beta-carotene is mainly located in the epidermis (45, 46) where UVB radiation largely is absorbed (47). Beta-carotene does not absorb in the UVB range of light, and the oxidative component of UVB light is only weak. In dermal areas, where less beta-carotene is located, its antioxidant activity might be more important as they are the major target of UVA-induced oxidative stress. Near-ultraviolet light even reaches the cutaneous capillary system, indicating a possible field of action for beta-carotene in this compartment.

Exposure to sunlight reduces levels of beta-carotene in skin (1, 48). However, a single UV treatment did not significantly change concentrations of beta-carotene in skin but lycopene levels were decreased (49, 50). Overall, reduction of levels of beta-carotene and other carotenoids in skin might lower protection against UV damage. Consequently it may be hypothesized that increasing of beta-carotene concentrations before exposure to sunlight might provide a surplus and thus should reduce the risk of photodamage. Hata *et al.* (45) reported a correlation between carotenoid levels in skin and skin cancer. They found significantly lower carotenoid concentrations within perilesional, actinic keratosis and basal cell carcinoma sites when compared to skin from healthy subjects, and they suggested that reduced carotenoid levels in skin might predispose to the development of skin cancer.

Moderate carotenodermia is widely considered as beautiful and healthy complexion, particularly in infants, and the use of high doses of beta-carotene is considered relatively nontoxic (51). Nevertheless, it should be considered that a "safe range" of intracellular beta-carotene levels has not been determined yet, and it is not known whether beta-carotene accumulation in the skin might exert harmful side effects.

BETA-CAROTENE IN THE PREVENTION OF SKIN PHOTODAMAGE

The experience that beta-carotene inhibits photosensitized reactions in human skin (EPP) seemed to justify studies on the photoprotective effect of beta-carotene in normal skin. Initially, the results from clinical studies were rather disappointing. In 1972, Mathews-Roth and coworkers (33) published results of a clinical trial showing that long-term oral supplementation with beta-carotene (180 mg/day) increases the MED (minimal erythemal dose) to a small but not significant extent. In other studies beta-carotene did not significantly protect against UVA, UVB, and PUVA erythema after single (120 mg/day) or chronic ingestion of beta-carotene (90–180 mg/day), although values of carotenoids in the plasma reached levels that had been shown to be protective in patients with EPP and although levels in skin increased accordingly and even manifested carotenodermia (49, 52).

A few clinical trials found significant preventive effects of beta-carotene against acute photodamage. For example, we reported reduced erythema formation in subjects which had been supplemented with beta-carotene (30 mg/day) for 10 weeks before and also during exposure to sunlight (2). In this study we also observed that beta-carotene significantly increased the density of Langerhans cells prior to sun exposure and prevented their decrease after irradiation. Long-term supplementation with beta-carotene (30 mg/day) also protected from UVA-induced immunosuppression, as determined by delayed-type hypersensitivity tests which are accepted assays to evaluate the individual sensitivity to UV light (2, 32, 53). Stahl *et al.* (17) reported significantly reduced erythema formation following carotenoid supplementation with mainly beta-carotene (25 mg/day) over 12 weeks. Additional supplementation with 335 mg of vitamin E per day increased the protective effect of beta-carotene, but not to a significant extent. Recently, Lee *et al.* (31) published data from individuals who had been supplemented over 24 weeks with increasing doses of carotenoids (30–90 mg/day) consisting of mainly beta-carotene. In these subjects, the MED rose with carotenoid intake to a small but significant extent. In contrast to our results a dose of only 30 mg/day carotenoids, however, did not significantly alter the MED. Besides, serum lipid peroxida-

tion, determined with a lipid peroxidation activity assay, was significantly diminished during carotenoid supplementation.

The effect of beta-carotene on erythema prevention should be considered rather modest. Besides, it might be questioned whether reduction or even complete suppression of erythema formation as a physiological response to overexposure to sunlight is indeed beneficial or desirable.

Regarding chronic manifestations of skin photodamage, there is a lack of data on the prevention of photoaging by beta-carotene. A few data on skin cancer prevention have been published, nevertheless, the results from observational studies are inconsistent (54–62), and data from randomized, controlled trials are scarce (34, 63). The Physicians' Health Study and its follow-up analysis showed that beta-carotene supplementation (50 mg/day) over a period of 12 years has no effect on the development of a first NMSC (34) and other malignant neoplasms (36). This corresponds to results from the Nambour Skin Cancer Prevention Trial, which showed that beta-carotene supplementation (30 mg/day) over 4–5 years does not alter incidence of a first NMSC, neither with nor without sunscreen use (35). Results from a few observational studies showed an association between increased beta-carotene levels in plasma and a reduced risk of a first NMSC (54, 56), but most studies found no such effect (55, 57–59, 64). Obviously it is at present not possible to determine whether there is a relationship between plasma beta-carotene levels and risk of a first NMSC.

Concerning secondary prevention of skin cancer, Greenberg *et al.* (63) conducted the only randomized controlled clinical trial on the effect of beta-carotene supplementation on NMSC development. Patients who had recently had NMSC were given beta-carotene (50 mg/day) over a period of 5 years. Though plasma levels of beta-carotene rose 10-fold, no protection against the development of a new skin cancer was found.

It may be concluded that the clinical data available do not clearly show any preventive effectiveness of beta-carotene supplementation on skin cancer.

BETA-CAROTENE FUNCTION IN SKIN EXPOSED TO SUNLIGHT

At present, the basic molecular and pathophysiological aspects of the interaction of beta-carotene, skin, and UV light are poorly understood. The absorption spectra of carotenoids typically occur in the near ultraviolet and visible light region of 360–550 nm (65); however, Sayre and Black (66) reported that even in yellowish skin not enough beta-carotene was present to filter hazardous radiation to a significant extent.

Furthermore, prevention of direct DNA damage by beta-carotene has been regarded less likely (66). What seems more feasible is that beta-carotene acts as an antioxidant in the skin. Beta-carotene might provide protection against photosensitized reactions by quenching triplet sensitizers and singlet oxygen by energy transfer. Furthermore, beta-carotene might react with ROS such as oxygen radicals, peroxyl radicals, and singlet oxygen (67–70). A variety of experimental studies investigated the antioxidant function of beta-carotene in the skin *in vivo* and *in vitro*. In rodents, beta-carotene was found to reduce lipid peroxidation (20, 71, 72), and topical application of beta-carotene reduced *in vivo* chemiluminescence (73, 74). It has also been demonstrated that beta-carotene quenches singlet oxygen-mediated photochemical reactions in rodent skin (75–77).

In cultured skin cells, a few *in vitro* studies have investigated the antioxidant potential of beta-carotene. Beta-carotene decreased photoinactivation of the enzymes catalase and superoxide dismutase, as well as protein cross linking (78). Furthermore, beta-carotene protected against membrane damage and lipid peroxidation (21). In rat kidney fibroblasts beta-carotene diminished UVA-induced catalase deactivation and lipid peroxidation (19), and in embryonic lung fibroblasts beta-carotene protected from UVA-induced cell damage (18). In this study, positive synergy effects were observed when beta-carotene treatment was combined with vitamin E or vitamins E plus C. Interestingly, treatment with vitamin E or C alone had no protective effects, and in cells exposed to UVB light, the protective effect of beta-carotene was minor.

It should be taken into consideration that beta-carotene might also act through one of its metabolites. Beta-carotene is likely to be degraded by photochemically generated ROS ("photobleaching") (79). Thus, the decrease observed in beta-carotene levels in skin and plasma might be due to photodegradation or photoisomerization. Whether such metabolites occur in the skin *in vivo* following exposure to solar radiation must be elucidated. Furthermore, as a provitamin A carotenoid, beta-carotene might be metabolized to retinoids via central cleavage by the enzyme 15,15′-dioxygenase, which has recently been found in mouse skin (80). Retinoic acid is considered the biologically most effective metabolite and it has been used successfully for prevention and treatment of skin photodamage (81–83). In human skin, expression of 15,15′-dioxygenase or formation of retinoic acid from beta-carotene has not been demonstrated yet.

Beside a variety of experimental data which it is claimed to explain beneficial or even preventive aspects of beta-carotene in skin, a few experimental data exist which document more or less detrimental effects. In mice, aggravating effects on skin cancer were found (84–86). For example, beta-carotene increased the formation of skin papillomas in mice (85) treated with

DMBA and TPA. Even so, beta-carotene inhibited the conversion of papillomas to carcinomas which indicates a chemopreventive effect of beta-carotene (85, 87). Black recently stated (84) that future studies using carotenoid supplementation should be carried out with caution until interactions of carotenoids and repair mechanisms of radicals are clarified.

We investigated the effect of beta-carotene on the cellular stress response in dermal fibroblasts on the level of gene expression. Using the UVA induction of heme oxygenase-1 (HO-1) as an accepted marker for oxidative stress (88), we studied the effect of beta-carotene (0.5 and 5.0 μM) on the HO-1 expression in irradiated cells. HO-1 induction is attenuated by cellular antioxidants. Accordingly, singlet oxygen quencher beta-carotene should diminish HO-1 induction in irradiated cells which is a consequence of photochemical generation of singlet oxygen (89). Unexpectedly, beta-carotene strongly enhanced the UVA induction of HO-1, which indicates that beta-carotene can have a prooxidative effect. In our study, the prooxidative effect of beta-carotene observed could be entirely suppressed by vitamin E (25 μM), but only moderately by vitamin C (100 μM) (37). As cosupplementation of the cells with vitamin E abolished the UV-induced increase of HO-1 in beta-carotene-treated cells, we assume that beta-carotene acts as a prooxidant and consequently causes membrane lipid peroxidation which can be prevented by vitamin E. It might be questioned whether this enhanced HO-1 expression could have beneficial effects, as HO-1 has been called an "emergency inducible defense pathway" for protection against UVA radiation in dermal fibroblasts (90). HO-1 is part of an adaptive response to UVA radiation which leads to protection against oxidative membrane damage (91) and mediates immunoprotection (92). According to present knowledge, induction of HO-1 is due to oxidative stress, e.g., UVA radiation, hydrogen peroxide, hypoxia, and hyperoxia. Our results show that beta-carotene may act as an amplifier of UVA-induced oxidative stress and subsequent increase of HO-1 expression.

Jones *et al.* (38) also published data demonstrating a prooxidative effect of beta-carotene in dermal fibroblasts which supports our findings. Beta-carotene (10 μM) was found to increase UVA/B-generated oxidative stress, resulting in increased release of superoxide anions and lipid peroxidation. Furthermore, beta-carotene reduced cellular adaptation to UV irradiation with a rise in catalase and superoxide dismutase activities and increase in cellular glutathione content.

CONCLUSIONS

Undoubtedly, beta-carotene is an important micronutrient with powerful biological effects. Nevertheless, clinical studies as a whole have failed to persuade of an important role of beta-carotene as a photoprotector. The lack of an effect on skin cancer prevention in clinical trials might result from inadequate study protocols, as the duration of trials might have been too short and secondary prevention not the right target. However, even the results on acute manifestations of photodamage were ambiguous and mostly weak. In contrast to its effectiveness in the treatment of photosensitivity in patients with EPP, beta-carotene does not seem to be clinically beneficial as an oral sunscreen for healthy subjects. Beta-carotene might function as an antioxidant in human skin, but increasing levels in skin seems unlikely to modify the severity of skin photodamage. Experimental studies on the mechanism of action in skin cells *in vitro* have raised many questions and opened up a wide field of future research on the role of beta-carotene as a skin anti- or prooxidant.

It seems reasonable to assume that beta-carotene might combine both beneficial and detrimental effects in skin exposed to sunlight. The effects might depend on the biological endpoint investigated and the concentration of other antioxidants, e.g., vitamin E. In terms of using beta-carotene as a skin photoprotectant it should be pointed out that oral supplementation with beta-carotene as a single antioxidant might lead to an imbalance in the cutaneous antioxidant network and thus, as documented, exert possibly harmful effects on skin exposed to sunlight.

It seems questionable whether the present knowledge on beta-carotene action is sufficient to recommend intake of oral beta-carotene supplements or fortified food. Actually, it has been stated that there is at present no scientific evidence that high nutritional intake of beta-carotene from fruits and vegetables or a low-dose oral supplementation with beta-carotene might be harmful *in vivo* in general (93). From the data presented, we conclude that the use of single beta-carotene supplementation as an oral sun protectant should not be recommended at this time.

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Protective Effects of Grape Seed Proanthocyanidins and Selected Antioxidants against TPA-Induced Hepatic and Brain Lipid Peroxidation and DNA Fragmentation, and Peritoneal Macrophage Activation in Mice

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ABSTRACT. 1. The comparative protective abilities of a grape seed proanthocyanidin extract (GSPE) (25–100 mg/kg), vitamin C (100 mg/kg), vitamin E succinate (VES) (100 mg/kg) and β-carotene (50 mg/kg) on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as production of reactive oxygen species by peritoneal macrophages, were assessed.

2. Treatment of mice with GSPE (100 mg/kg), vitamin C, VES and β-carotene decreased TPA-induced production of reactive oxygen species, as evidenced by decreases in the chemiluminescence response in peritoneal macrophages by approximately 70%, 18%, 47% and 16%, respectively, and cytochrome c reduction by approximately 65%, 15%, 37% and 19%, respectively, compared with controls.

3. GSPE, vitamin C, VES and β-carotene decreased TPA-induced DNA fragmentation by approximately 47%, 10%, 30% and 11%, respectively, in the hepatic tissues, and 50%, 14%, 31% and 11%, respectively, in the brain tissues, at the doses that were used. Similar results were observed with respect to lipid peroxidation in hepatic mitochondria and microsomes and in brain homogenates.

4. GSPE exhibited a dose-dependent inhibition of TPA-induced lipid peroxidation and DNA fragmentation in liver and brain, as well as a dose-dependent inhibition of TPA-induced reactive oxygen species production in peritoneal macrophages.

5. GSPE and other antioxidants provided significant protection against TPA-induced oxidative damage, with GSPE providing better protection than did other antioxidants at the doses that were employed. GEN PHARMAC 30;5:771–776, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Oxidative stress, lipid peroxidation, DNA fragmentation, grape seed proanthocyanidin extract, vitamin C, vitamin E succinate, β-carotene, zinc L-methionine, Swiss-Webster mice, 12-O-tetradecanoylphorbol-13-acetate (TPA)

INTRODUCTION

Free radicals have been implicated in more than 100 disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, aging, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, tumor promotion and carcinogenesis, and AIDS (Ames, 1992; Halliwell, 1996; Halliwell and Cross, 1991; Halliwell *et al.*, 1992; Kehrer, 1993; Pitot and Dragan, 1991). Free radicals and their metabolites are increasingly recognized for their contribution to tissue injury leading to both initiation and promotion of multistage carcinogenesis (Pitot and Dragan, 1991). Recent studies have demonstrated that environmental pollutants, radiation, pesticides, various medications, contaminated water and deep-fried and spicy foods, as well as physical stress, exhibit the ability to produce enormous amounts of free radicals, resulting in oxidative deterioration of lipids, proteins and DNA, activation or procarcinogens, inhibition of cellular and antioxidant defense systems, depletion of sulfhydryls, altered calcium homeostasis, changes in gene expression and induction of abnormal proteins (Ames, 1992; Halliwell, 1996; Kehrer, 1993; Stohs and Bagchi, 1993).

Antioxidants/free-radical scavengers function as inhibitors at both initiation and promotion/propagation/transformation stages of tumor promotion/carcinogenesis and protect cells against oxidative damage (Halliwell, 1996; Halliwell and Cross, 1991; Halliwell *et al.*, 1992). The consumption of edible plants, fruits and vegetables has been demonstrated to prevent the occurrence of a number of diseases in humans and animals (Hocman, 1989). Vegetables, fruits and their seeds are rich sources of vitamins C and E, β-carotene and protease inhibitors, compounds that might protect the organism against free radical–induced injury and diseases (Hocman, 1989).

Proanthocyanidins, naturally occurring compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark, are a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics. Proanthocyanidins have been reported to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic and vasodilatory actions (Afanas'ev *et al.*, 1989; Buening *et al.*, 1981; Kolodziej *et al.*, 1995). Furthermore, proanthocyanidins have been reported to inhibit lipid peroxidation, platelet aggregation and capillary perme-

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Exhibit B

ability and fragility and to modulate the activity of enzyme systems including cyclooxygenase and lipooxygenase (Bors and Saran, 1987; Kolodziej *et al.*, 1995). Proanthocyanidins are believed to be nontoxic. If they are absorbed and biologically active *in vivo*, they may prevent free radical–mediated cytotoxicity and lipid peroxidation and protect low-density lipoproteins from oxidation (Frankel *et al.*, 1993; Kinsella *et al.*, 1993).

A variety of proanthocyanidins have been shown to prevent the growth of breast cancer cells and to inhibit the enzymes taking part in the replication of rhino viruses (common cold) and HIV viruses (Hocman, 1989). The potential of isoflavones and lignans, also known as phytoestrogens, for preventing the development of hormone-dependent cancers such as breast and prostate cancer is attributed to their being weak estrogens (Hocman, 1989). Proanthocyanidins may exert these effects as antioxidants, potent free-radical scavengers and chelators of toxic heavy metals (Chen *et al.*, 1996; Rice-Evans *et al.*, 1996).

In this study, we have assessed the comparative protective abilities of a grape seed proanthocyanidin extract (GSPE) with vitamin C, vitamin E succinate (VES) and β-carotene *in vivo* against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced lipid peroxidation and DNA fragmentation in hepatic and brain tissues as well as against production of oxygen free radicals in peritoneal macrophages of mice.

MATERIALS AND METHODS

Chemicals

A commercially available dried, powdered GSPE (batch no. AV 609016) was obtained from InterHealth Nutritionals Inc. (Concord, CA). All other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or the highest grade available.

Animals and treatment

Female Swiss-Webster mice (20–25 g) were obtained from Sasco (Omaha, NE). The animals were housed in a controlled environment at 25°C with a 12-hr light and 12-hr dark cycle and were acclimated for at least 3–5 days before use. All animals were allowed free access to food (Purina Rodent Lab Chow No. 5001) and tap water. VES and β-carotene were dissolved in corn oil, whereas GSPE and vitamin C were dissolved in water. GSPE (25–100 mg/kg), vitamin C (100 mg/kg), VES (100 mg/kg) and β-carotene (50 mg/kg) were orally administered to groups of animals with the use of a feeding needle for 7 consecutive days. All treatments were conducted daily in the morning between 7:30 A.M. and 8:30 A.M. All groups of mice received an intraperitoneal (IP) injection of 1 ml of 3% thioglycollate (DIFCO Laboratories, Detroit, MI) broth 3 days before TPA treatment to elicit peritoneal macrophages (Witz and Czerniecki, 1989). TPA was administered on the 8th day 2 hr after the antioxidant treatment. Groups of mice were individually treated IP with 0.1 μg TPA diluted in 1 ml of sterile phosphate-buffered saline (PBS) to induce an oxidative stress and were killed 2 hr posttreatment by cervical dislocation. Control animals received the PBS buffer. The peritoneal macrophage cells were isolated, the hepatic and brain tissues were quickly removed and the subcellular fractions were obtained as described elsewhere (Bagchi and Stohs, 1993). An approval (ARC no. 0313) from the Creighton University Animal Research Committee was obtained for this project.

Chemiluminescence assay

Chemiluminescence, as an index of reactive oxygen species production, was measured in a Chronolog Lumivette luminometer (Chronolog Corp., Philadelphia, PA). The assay was conducted in 3-ml glass minivials. The vials were incubated at 37°C before measurement, and the background chemiluminescence of each vial was checked before use. Samples were preincubated at 37°C for 15 min, and 4 μM luminol was added to enhance chemiluminescence. All additions to the vials as well as chemiluminescence-counting procedures were performed under dim lighting conditions. Results were presented as counts per unit time minus background. Chemiluminescence was monitored for 6 min at continuous 30-sec intervals (Bagchi and Stohs, 1993).

Cytochrome c reduction assay

Superoxide anion production by peritoneal macrophages was measured by the cytochrome c reduction assay of Babior *et al.* (1973). The reaction mixtures contained 1 ml of macrophages (3×10^6 cells/ml) and 0.05 mM cytochrome c. The reaction mixtures were incubated for 15 min at 37°C. The reactions were terminated by placing the reaction mixtures in ice. The mixtures were centrifuged at 1,500*g* for 10 min at 4°C, and the supernatant fractions were transferred to clean tubes for subsequent spectrophotometric measurement at 550 nm. Absorbance values were converted into nanomoles of cytochrome c reduced by using the extinction coefficient of 2.1×10^4/M/cm/15 min (Bagchi and Stohs, 1993).

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) associated with hepatic mitochondria and microsomes, as well as brain homogenates from control and treated animals were determined as an index of lipid peroxidation according to the method of Buege and Aust (1984) and as previously published by us (Bagchi and Stohs, 1993). Malondialdehyde was used as the standard. Absorbance values were measured at 535 nm, and an extinction coefficient of 1.56×10^5/M/cm was used.

DNA fragmentation

Liver and brain samples were homogenized in lysis buffer (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were centrifuged at 27,000*g* for 20 min to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 N perchloric acid, and 5.5 N perchloric acid was added to supernatant samples to reach a concentration of 0.5 N. Samples were heated at 90°C for 15 min and centrifuged at 1,500*g* for 10 min to remove protein. Resulting supernatants were reacted with diphenylamine for 16–20 hr at room temperature, and absorbance was measured at 600 nm. DNA fragmentation is expressed as a percentage of total DNA appearing in the supernatant fractions. Treatment effects are reported as a percentage of control fragmentation (Ray *et al.*, 1993).

Statistical analysis

Data for each group were subjected to analysis of variance. Scheffe's S method was used as the *post hoc* test. The data are expressed as the mean±standard deviation of four animals. The level of statistical significance employed in all cases was $P<0.05$.

RESULTS

Production of reactive oxygen species

TPA-induced *in vivo* production of oxygen free radicals in the peritoneal macrophages of mice was assessed by luminol-enhanced chemiluminescence and cytochrome c reduction assays. The results

TABLE 1. Production of reactive oxygen species by peritoneal macrophages based on chemiluminescence response and cytochrome c reduction after treatment of mice with TPA, and the comparative scavenging abilities of GSPE and selected antioxidants

Sample	Chemiluminescence (CPM/3 × 10^6 cells)	Cytochrome c reduction (nmoles/15 min/3 × 10^6 cells)
Control	995 ± 156a	4.55 ± 0.43a
Corn oil	937 ± 88a	4.35 ± 0.17a
Vitamin C (100 mg/kg)	1114 ± 141a	5.27 ± 0.50b
VES (100 mg/kg)	1771 ± 139b	9.10 ± 0.65c
Vitamin C + VES (100 mg/kg each)	1724 ± 140b	9.02 ± 0.58c
β-Carotene (50 mg/kg)	1198 ± 118a	3.93 ± 0.70a
GSPE (100 mg/kg)	1306 ± 94a	5.07 ± 0.58b
TPA	6031 ± 591c	26.61 ± 1.60d
TPA + vitamin C (100 mg/kg)	5081 ± 335d	22.67 ± 2.36d
TPA + VES (100 mg/kg)	3455 ± 321e	18.03 ± 0.83e
TPA + vitamin C + VES (100 mg/kg each)	2934 ± 132e	14.24 ± 1.52f
TPA + β-carotene (50 mg/kg)	5015 ± 199d	21.81 ± 1.38d
TPA + GSPE (25 mg/kg)	3592 ± 211e	18.21 ± 1.86e
TPA + GSPE (50 mg/kg)	2732 ± 99e	12.65 ± 1.41f
TPA + GSPE (100 mg/kg)	1724 ± 268b	8.26 ± 0.84c

Female Swiss-Webster mice were treated with a single dose of 0.1 μg TPA after treatment with antioxidant(s) for 7 days. Peritoneal exudate cells (primarily macrophages) were analyzed for enhanced chemiluminescence and cytochrome c reduction. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

of the chemiluminescence and cytochrome c reduction assays for the production of reactive oxygen species by peritoneal exudate cells (primarily macrophages) are presented in Table 1.

The chemiluminescence produced by peritoneal macrophages from TPA-treated animals rapidly rises, reaching a maximum between 3 and 4 min, whereas macrophages from control animals reach a peak chemiluminescence at 3 min (data not shown). No significant increases in chemiluminescence were observed after treatment of the mice with either GSPE, vitamin C or β-carotene (Table 1). An approximately 1.8-fold increase in chemiluminescence was observed in the peritoneal macrophages of animals treated with VES alone. The succinate moiety has been previously shown to be responsible for this effect (Bagchi *et al.*, 1993).

A 6.1-fold increase in chemiluminescence was observed after treatment of the animals with TPA. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 consecutive days decreased the TPA-induced chemiluminescence in the peritoneal macrophages by 40%, 55% and 71%, respectively, compared with control values. Thus, a dose-dependent inhibition was demonstrated by GSPE. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased the TPA-induced chemiluminescence by 16%, 43%, 51% and 71%, respectively, compared with control samples. Administration of β-carotene (50 mg/kg) and GSPE (50 mg/kg) for 7 consecutive days decreased the TPA-induced chemiluminescence response by 17% and 55%, respectively, relative to the control values.

The effect of TPA on the production of superoxide anion by peritoneal macrophages (determined by the cytochrome c reduction assay) also is presented in Table 1. The data are expressed as nanomoles of cytochrome c reduced 3×10^6 cells/15 min. GSPE, vitamin C and β-carotene had no effect on superoxide anion production in the absence of TPA. As previously noted with chemiluminescence, VES produced a significant increase (approximately 2.0-fold) in superoxide anion production (Table 1).

TPA administration increased the production of superoxide anion on the basis of cytochrome c reduction compared with the cells from untreated animals by 5.9-fold (Table 1). GSPE induced a dose-dependent inhibition of the TPA-induced cytochrome c reduction. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 consecutive days decreased TPA-induced cytochrome c reduction by 32%, 53% and 69%, respectively, which still represented approximately 4.0-, 2.8- and 1.8-fold increases, respectively, above the control values. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased TPA-induced cytochrome c reduction by approximately 15%, 32%, 47% and 69%, respectively, compared with control samples. Administration of β-carotene (50 mg/kg) and GSPE (50 mg/kg) for 7 consecutive days decreased TPA-induced cytochrome c reduction by approximately 18% and 48%, respectively, relative to the control values.

Lipid peroxidation

The effects of TPA and antioxidants on lipid peroxidation in hepatic mitochondria and microsomes and in brain homogenates are summarized in Table 2. No significant increases in lipid peroxidation were observed with GSPE, vitamin C or β-carotene. Approximately 1.4-, 1.2- and 1.2-fold increases in lipid peroxidation were observed in the hepatic mitochondria, hepatic microsomes, and brain homogenates, respectively, compared with control animals after treatment of the animals with VES, similar to previously reported observations (Bagchi *et al.*, 1993).

After treatment of mice with TPA, increases in lipid peroxidation of 2.7-, 2.9- and 3.1-fold were observed in hepatic mitochondria, hepatic microsomes and brain homogenates, respectively, compared with control values. Administration of 25, 50 and 100 mg GSPE/kg for 7 days to these animals decreased TPA-induced hepatic mitochondrial lipid peroxidation by 37%, 41% and 46%, respectively; in the hepatic microsomal fractions, decreases of 47%, 55% and 59% were observed, respectively, compared with control values. Approximately 46%, 53% and 61% decreases were demonstrated by GSPE

TABLE 2. TPA-induced lipid peroxidation in hepatic mitochondria and microsomes, and in brain homogenates of mice, and the comparative protective abilities of GSPE and selected antioxidants

	Lipid peroxidation (nmoles MDA/mg of protein)		
	Liver		Brain
	Mitochondria	Microsomes	Whole homogenate
Control	2.17 ± 0.24a	2.76 ± 0.22a	1.62 ± 0.13a
Corn oil	2.30 ± 0.11a	2.59 ± 0.35a	1.69 ± 0.13a
Vitamin C (100 mg/kg)	2.38 ± 0.19a	2.85 ± 0.25a	1.68 ± 0.10a
Vitamin E succinate (VES) (100 mg/kg)	3.05 ± 0.14b	3.19 ± 0.29b	1.93 ± 0.17b
Vitamin C + (VES) (100 mg/kg each)	3.01 ± 0.17b	3.11 ± 0.15b	1.95 ± 0.10b
β-Carotene (50 mg/kg)	2.11 ± 0.12a	2.83 ± 0.11a	1.55 ± 0.12a
GSPE (100 mg/kg)	2.32 ± 0.14a	2.67 ± 0.32a	1.71 ± 0.22a
TPA	5.81 ± 0.34c	8.12 ± 0.84c	4.95 ± 0.32c
TPA + Vitamin C (100 mg/kg)	5.12 ± 0.34c	7.02 ± 0.42c	4.32 ± 0.23c
TPA + VES (100 mg/kg)	3.71 ± 0.39d	4.29 ± 0.44d	2.71 ± 0.49d
TPA + Vitamin C + VES (100 mg/kg each)	3.54 ± 0.49b,d	3.81 ± 0.42d,e	2.57 ± 0.35d
TPA + β-Carotene	5.41 ± 0.38c	7.17 ± 0.46c	4.54 ± 0.22c
TPA + GSPE (25 mg/kg)	3.68 ± 0.39d	4.33 ± 0.49d	2.69 ± 0.28d
TPA + GSPE (50 mg/kg)	3.43 ± 0.22d	3.65 ± 0.25e	2.33 ± 0.24d
TPA + GSPE (100 mg/kg)	3.13 ± 0.27b	3.31 ± 0.28b	1.94 ± 0.40b

Swiss-Webster mice were treated with a single dose TPA after receiving antioxidant(s) for 7 days. Thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation were determined on hepatic mitochondria and microsomes and on brain homogenates from control and treated animals. Malondialdehyde was used as the standard. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

against TPA-induced lipid peroxidation in the brain homogenates at these same concentrations.

Administration of vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C and VES (100 mg/kg each) and GSPE (100 mg/kg) for 7 days decreased TPA-induced hepatic mitochondrial lipid peroxidation by 12%, 36%, 39% and 46%, respectively, compared with control values, and 14%, 47%, 53% and 59% decreases, respectively, were observed in the hepatic microsomes. After treatment of the animals with these same antioxidants, 13%, 45%, 48% and 61% decreases, respectively, were observed against TPA-induced lipid peroxidation in brain homogenates. Administration of β-carotene (50 mg/kg) decreased TPA-induced hepatic mitochondrial and microsomal lipid peroxidation by approximately 7% and 12%, respectively; under these same conditions, an 8% decrease was observed in brain homogenate lipid peroxidation, compared with control values.

DNA fragmentation

Programmed cell death (apoptosis) has been identified as a selective process of physiological cell deletion. Apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, condensation and fragmentation of nuclei and extensive degradation of chromosomal DNA. Fragmentation of nuclear DNA is a biochemical hallmark of apoptosis (Schwartzman and Cidlowski, 1993).

TPA-induced DNA fragmentation in hepatic and brain tissues is summarized in Table 3, and the comparative protective abilities of various antioxidants are presented. TPA-induced 2.2- and 2.5-fold increases in DNA fragmentation in the hepatic and brain tissues of mice, respectively, compared with controls. No significant increases in DNA fragmentation were observed with GSPE, vitamin C or β-carotene. Approximately 1.3- and 1.4-fold increases in DNA fragmentation were observed in the liver and brain tissues, respectively, compared with control animals after treatment of the animals with VES alone, similar to previous observations (Bagchi *et al.*, 1993).

A dose-dependent protective ability against TPA-induced DNA fragmentation was demonstrated by GSPE. Administration of 25, 50 and 100 mg GSPE/kg to the animals for 7 days decreased TPA-induced hepatic DNA fragmentation by 36%, 42% and 47%, respectively, compared with control values, and DNA fragmentation decreased by approximately 32%, 44% and 50% in the brain tissues at these same concentrations. Pretreatment of animals with vitamin C (100 mg/kg), VES (100 mg/kg), a combination of vitamin C plus VES (100 mg/kg each) and GSPE (100 mg/kg) decreased TPA-induced hepatic DNA fragmentation by 10%, 30%, 38% and 47%, respectively; under these same conditions, DNA fragmentation was reduced by 14%, 31%, 40% and 50% in brain tissues, respectively, compared with control samples. Administration of β-carotene (50 mg/kg) for 7 days reduced TPA-induced hepatic and brain DNA fragmentation by 11%, relative to the respective control values.

DISCUSSION

Proanthocyanidins are a group of polyphenolic bioflavonoids ubiquitously found in fruits and vegetables. Proanthocyanidins have gained recent interest because of their broad pharmacological activity and therapeutic potential (Chen *et al.*, 1996; Hanefield and Herrmann, 1976; Masqueliér *et al.*, 1979). Putative therapeutic effects of many traditional medicines may be ascribed to the presence of bioflavonoids (Brandi, 1992; Chen and Chan, 1996; Havsteen, 1983). The chemical properties of bioflavonoids in terms of the availability of the phenolic hydrogens as hydrogen-donating radical scavengers and singlet oxygen quenchers predict their antioxidant activity (Chen *et al.*, 1996; Rice-Evans *et al.*, 1996). For a proanthocyanidin or a bioflavonoid to be defined as an antioxidant, it must satisfy two basic conditions: (1) when present in low concentrations relative to the substrate to be oxidized, it can delay, retard, or pre-

TABLE 3. TPA-induced DNA fragmentation in the hepatic and brain tissues, and the comparative protective abilities of GSPE and selected antioxidants

Sample	Liver (%)	Brain (%)
Control	2.04 ± 0.30a	1.77 ± 0.28a
Corn oil	2.19 ± 0.31a	1.73 ± 0.22a
Vitamin C (100 mg/kg)	2.16 ± 0.34a	2.19 ± 0.31a,b
VES (100 mg/kg)	2.63 ± 0.24b	2.47 ± 0.36b
Vitamin C + VES (100 mg/kg each)	2.54 ± 0.36b	2.33 ± 0.31b
β-Carotene (50 mg/kg)	2.13 ± 0.22a	1.97 ± 0.26a
GSPE (100 mg/kg)	2.16 ± 0.31a	1.89 ± 0.31a
TPA	4.57 ± 0.51c	4.41 ± 0.28c
TPA + vitamin C (100 mg/kg)	4.12 ± 0.31c	3.80 ± 0.38d
TPA + VES (100 mg/kg)	3.18 ± 0.45d	3.03 ± 0.26e
TPA + vitamin C + VES (100 mg/kg each)	2.83 ± 0.23b,d	2.66 ± 0.21b
TPA + β-carotene (50 mg/kg)	4.06 ± 0.29c	3.92 ± 0.20d
TPA + GSPE (25 mg/kg)	2.94 ± 0.51b,d	3.00 ± 0.16c
TPA + GSPE (50 mg/kg)	2.67 ± 0.21b	2.49 ± 0.24b
TPA + GSPE (100 mg/kg)	2.43 ± 0.21b	2.22 ± 0.19b

Female Swiss-Webster mice were treated with a single dose of 0.1 μg TPA after receiving antioxidant(s) for 7 days. DNA fragmentation was measured spectrophotometrically by using Burton's reagent. Each value represents the mean ± SD of four mice. Values with nonidentical lowercase letters are significantly different ($P < 0.05$).

vent autooxidation or free radical–mediated oxidative injury; and (2) the resulting product formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation (Shahidi and Wanasundara, 1992).

The biological, pharmacological and medicinal properties of the bioflavonoids and proanthocyanidins have been extensively reviewed (Jovanovic *et al.*, 1994; Rice-Evans *et al.*, 1996; Suzuki, 1993). Flavonoids and other plant phenolics are reported to possess, in addition to their free-radical scavenging and antioxidant activity, multiple biological activities including vasodilatory, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, antiallergic, antiviral and estrogenic activities, as well as being inhibitors of the enzymes phospholipase A_2, cyclooxygenase and lipooxygenase (Rice-Evans *et al.*, 1996; Salah *et al.*, 1995).

The presence of various phenolic compounds, including phenoldienones, epicatechin, epigallocatechin, epigallocatechin gallate, ferulic acid, caffeic acid, *p*-coumaric acid, kaempferol, quercetin and myricetin, have been well established in proanthocyanidin extracts (Gonzalez *et al.*, 1982; Hanefield and Herrmann, 1976; Masquelier *et al.*, 1979; Rice-Evans *et al.*, 1996). It is well known that diets rich in fresh fruits and vegetables are protective against cardiovascular diseases and other oxidative stress–induced diseases and disorders including cancer (Chen and Chan, 1996; Halliwell, 1996; Halliwell *et al.*, 1992; Hocman, 1989). These chemoprotective properties have been attributed, in large part, to the presence of antioxidant nutrients vitamin C, vitamin E, β-carotene and mineral micronutrients. However, plant phenolics such as the bioflavonoids, proanthocyanidins and phenylpropanoids also may play a significant role. The proanthocyanidins or polyphenolic bioflavonoids may act as antioxidants or by other mechanisms, contributing to anticarcinogenic or chemoprotective actions or both.

In this study, the protective abilities of GSPE, a commercially available grape seed proanthocyanidin extract, vitamin C, VES, a combination of vitamin C plus VES and β-carotene were assessed on TPA-induced oxidative tissue and DNA damage in the hepatic and brain tissues, as well as activation of peritoneal macrophages. The production of reactive oxygen species by peritoneal macrophages was assessed by measuring chemiluminescence and cytochrome c reduction (Table 1). Cytochrome c reduction is a specific test for superoxide anion production (Ritchey *et al.*, 1981), whereas chemiluminescence is a general assay for the production of reactive oxygen species (Fisher and Adams, 1985). These assays clearly demonstrate the production of reactive oxygen species by peritoneal macrophages after administration of TPA and the comparative protective abilities of GSPE, vitamin C, a combination of vitamin C plus VES and β-carotene. GSPE demonstrated the best protection in the chemiluminescence assay compared with vitamin C, VES or β-carotene at the doses that were used. The combination of vitamin C and VES demonstrated better protection compared with the individual vitamins alone, which may be the result of regeneration of vitamin E from its oxidized form by vitamin C (Buettner, 1993). Similar results were obtained in the cytochrome c reduction assay (Table 1). These data indicate that GSPE as well as other antioxidants may be useful in preventing the *in vivo* production of reactive oxygen species.

Lipid peroxidation was assessed in the hepatic mitochondria and microsomes and in brain homogenate (Table 2); DNA fragmentation data for hepatic and brain tissues are presented in Table 3. Lipid peroxidation and DNA fragmentation serve as indicators of oxidative tissue damage. DNA fragmentation is believed to be a biochemical hallmark of apoptosis (programmed cell death), which plays a major role in developmental biology and in the maintenance of homeostasis in vertebrates (Schwartzman and Cidlowski, 1993). GSPE exhibited the best protection against TPA-induced hepatic mitochondrial and microsomal lipid peroxidation compared with the other antioxidants tested at the doses that were used (Table 2). A combination of vitamin C plus VES exerted better protection than did the corresponding individual vitamins. All antioxidants that were tested ameliorated TPA-induced increases in lipid peroxidation and DNA fragmentation in both brain and liver (Tables 2 and 3), with GSPE exhibiting the best protection compared with the other antioxidants.

These *in vivo* experiments demonstrate that GSPE is a better scavenger of free radicals and inhibitor of oxidative tissue damage than vitamin C, VES, a combination of vitamin C plus VES and β-carotene at the doses that were used. The results clearly demon-

strate that GSPE significantly attenuates TPA-induced oxidative stress in hepatic and brain issues, as well as in peritoneal exudate cells (primarily macrophages). These data confirm that GSPE can significantly attenuate TPA-induced lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as the enhanced production of oxygen free radicals in peritoneal macrophages. Furthermore, the results indicate that GSPE is bioavailable to vital target organs, including the liver and brain tissues and peritoneal exudate cells, and may therefore be useful in preventing the production of reactive oxygen species and oxidative tissue damage *in vivo*.

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